Postharvest Biology and Technology 49 (2008) 330–339

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Characterization of cultivar differences in alcohol acyltransferase and 1-aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening

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ARTICLE INFO

Article history:
Received 10 January 2008
Accepted 26 March 2008

Keywords:
Malus domestica (Borkh.)
Volatile esters
Ethylene
1-Methylcyclopropene
Fruit ripening

ABSTRACT

Alcohol acyltransferase (AAT) catalyzes the last step of volatile ester biosynthesis, and ethylene purportedly regulates AAT gene expression. In this study, expression patterns of apple (*Malus* × *domestica* Borkh.) AAT genes and ethylene biosynthesis genes of 1-aminocyclopropane-1-carboxylate synthase (ACS) were investigated in cultivars with relatively high ('Golden Delicious') or low ('Granny Smith') volatile ester production. All four AAT genes expressed stronger in 'Golden Delicious' than in 'Granny Smith'. MdAAT1 and MdAAT2 are the predominant genes expressed in fruit tissues. The expression levels of MdAAT1 and MdAAT2 were increasing as ripening progressed and were consistent with the total amount of esters detected between two cultivars. The transcript levels of MdAAT3 and MdAAT4 decreased at or after the onset of ripening. The expression of MdACS1 was significantly increased at the onset of ripening in both cultivars, while the expression of MdACS3 was detected throughout the harvest period in 'Golden Delicious'. Postharvest methylcyclopropene (1-MCP) exposure had little impact on expression of MdAAT1 and MdACS3 genes, but substantially suppressed the transcript level for MdACS1 in both cultivars, and MdAAT2 in 'Golden Delicious'. The results indicated that (1) differential expression of AAT genes may contribute to phenotypic variation of volatile ester biosynthesis, and (2) the expression of MdACS3 may play a role on induction of AAT genes expression in early fruit development, because it was expressed prior to ACS1. (3) The climacteric expression of MdACS1 greatly enhanced the expression levels of MdAAT1 and MdAAT2 genes and the emission of aromatic volatile esters. (4) Postharvest 1-MCP treatment showed selected inhibition on gene expression of specific AAT and ACS family members.

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1. Introduction

Plants produce a characteristic volatile compound profile resulting in a unique scent complex. These compounds serve as cues that facilitate a variety of biological functions including attraction of pollinators and seed dispersers, repelling herbivorous insects, and recruiting predators of insect herbivores (Pichersky and Gershenzon, 2002; Gang, 2005). The volatile profile of apple (*Malus* × *domestica* Borkh.) fruit is comprised of many compounds including esters, alcohols, aldehydes, ketones, terpenes, and ethers (Dimick and Hoskin, 1983), with esters accounting for up to 80–98% of total volatile production during ripening (Flath et al., 1967; López et al., 1998b). Apple fruit volatile esters contribute to fruit quality and influence consumer acceptance (Daillant-Spinnler et al., 1996; Dever and Cliff, 1995; Dixon and Hewett, 2000; Harker et al., 2003).

Alcohol acyltransferase (AAT, EC 2.3.1.84) catalyzes reactions between various alcohols and acyl-CoAs to produce esters (Harada et al., 1985; Aharoni et al., 2000; Shalit et al., 2003). AAT belongs to the BAHD super-family of acyltransferases, and apple AATs are categorized in subgroup 5 (Gang, 2005). The primary products of subgroup 5 are short to medium chain-length esters (D'Auria, 2006; Souleyre et al., 2005), for example, the substrates for apple MdAAT1 include C3 to C10 alcohols and C3, C6 and C8 acyl-CoAs (Souleyre et al., 2005; Li et al., 2006). Besides the reported full-length cDNA, the Genome Database for Rosaceae, Malus unigene version 2 (GDR, http://www.bioinfo.wsu.edu/gdr/), lists 3 AAT unigenes and several singlets.

The genetic basis regulating cultivar differences in volatile ester production, including the relative contribution of each member of the AAT gene family, has not been characterized. The concentration and composition of volatile esters in fresh and stored apples can be affected by several factors including harvest maturity (Sapers et al., 1977; Yahia et al., 1990) and storage regime (Patterson et al., 1974; Streif and Bangerth, 1988). However, phenotypic differences in volatile production are apparently caused by the plant genotype, as the qualitative and quantitative differences in volatile ester production are significant among apple cultivars (Fellman et al., 2000;

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0925-5214/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.postharvbio.2008.03.015

Holland et al., 2005; López et al., 1998a). The availability of substrate and the differential substrate specificities of AAT enzymes could also contribute to cultivar differences in volatile ester production (Holland et al., 2005; Echeverría et al., 2004). Relationships between expression patterns of AAT gene family members and cultivar-specific volatile ester production needs to be defined as AAT purportedly is the rate-limiting step in apple ester biosynthesis (Defilippi et al., 2005b). It is reported that there is distinct substrate specificity of AAT enzymes between 'Fuji' and 'Granny Smith', a differences likely to be the consequences of variations from multiple gene products (Holland et al., 2005). Apple volatile production occurs primarily in the peel (Guadagni et al., 1971; Knee and Hatfield, 1981) where the MdAAT2 protein has been immunolocalized (Li et al., 2006). Correlations between AAT gene expression levels and volatile ester production among elite strawberry cultivars have been reported (Carbone et al., 2006).

Ethylene imparts considerable control of apple fruit ripening, and it regulates apple volatile ester production via its impact on AAT gene expression (Li et al., 2006; Defilippi et al., 2005a; Schaffer et al., 2007). Expression of 1-aminocyclopropane-1-carboxylate synthase (ACS) genes is required for ethylene production (Yang and Hoffman, 1984; Harada et al., 2000), and at least two ACS genes, MdACS1 and MdACS3, are expressed in apple fruit tissues (Wiersma et al., 2007). Different ACS1 allelotypes significantly influence the ethylene production capacity and apple fruit textural attributes (Oraguzie et al., 2007; Zhu and Barritt, 2008). 1-Methylcyclopropene (1-MCP), an ethylene action inhibitor, inhibits ripening of apple fruit including the production of ripening-related volatile compounds (Fan et al., 1998; Li et al., 2006). However, the relationship between expression of individual ACS gene family members, volatile ester production, and the role of ethylene action in AAT gene expression has yet to be elucidated.

'Golden Delicious' and 'Granny Smith' represent two phenotypic extremes in volatile compound production by apples (López et al., 1998a; Holland et al., 2005; Li et al., 2006). The objective of this study was to characterize the expression patterns of AAT and ACS gene family members and examine the relationship with volatile ester production during on-tree development and postharvest ripening.

2. Materials and methods

2.1. Fruit and tissue sample collection

Fruit of two apple (*Malus* × *domestica* Borkh.) cultivars, 'Golden Delicious' and 'Granny Smith', were harvested from a research orchard near Orondo, WA during the 2006 harvest season. Fruit with uniform size and appearance were harvested weekly beginning approximately 4 weeks before and continued 4 weeks after commercial maturity as defined by starch pattern index, fruit firmness, internal ethylene concentration (IEC), and respiration rate as described previously (Argenta et al., 2002). Fruit firmness was measured on pared fruit surfaces using a Mohr Digitest instrument (Mohr and Associates, Richland, WA). For gene expression analysis, sampling dates were designated as 0 for commercial maturity, i.e. GS0 and GD0 for 'Granny Smith' and 'Golden Delicious' respectively, −4 and −2 for 4 and 2 weeks prior to 0, and +2 and +4 for 2 and 4 weeks after commercial maturity. Gene expression during postharvest ripening was assessed from samples collected from fruit harvested at commercial maturity and held at 20 °C for 6 weeks. Half of these fruit were treated the day of harvest with the ethylene action inhibitor 1-methylcylclopropene (1-MCP) (Mattheis et al., 2005). Volatile compounds were sampled at harvest and at 2-week intervals during postharvest ripening. Peel tissue was excised using a hand peeler, and immediately frozen in liquid nitrogen. Frozen peel was stored at −80 °C until it was used for analysis.

2.2. Analysis of volatile compounds

Volatile compounds were collected using dynamic headspace sampling from intact fruit (3 replicates). Depending on fruit size, apples were enclosed in 1 or 4-L glass jars with Teflon lids that were continuously purged at 1.67 mL s^{-1} with purified compressed air. Headspace components were adsorbed from the outlet stream onto 50 mg of 0.51–0.85 mm Tenax TA packed in glass tubing (17.5 cm × 0.4 cm i.d.). Tubes were desorbed at 250 °C for 3 min in He and the analytes condensed on a cryofocusing module adjusted to −120 °C using a Tekmar 6016 aero trap desorber (Tekmar Co., Cincinnati, OH)., The inlet was flash-heated to 250 °C to release the focused analytes into a Hewlett-Packard 5890A/5971A GC-MS equipped with a DB-Wax column (J&W Scientific, Folsom, CA, USA; 60 m × 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was held at 35 °C for 5 min, then increased to 50 °C at 0.033 °C s^{-1} and, finally, to 200 °C at the rate of 0.083 °C s^{-1} and held for 5 min. The linear velocity of the He carrier gas was 30 cm s^{-1}. Transfer line and ion source temperatures were 280 and 180 °C, respectively. Mass spectra were obtained using electron ionization at 70 eV. All compounds were identified using spectral and retention index comparison with authentic standards as well as the NIST05 library. Quantification was performed by comparing the area under target ion peaks from known amounts of standard with corresponding sample components using Chemstation and the QEDIT macro (Agilent, Palo Alto, CA, USA). Values were regressed against harvest date or storage duration using SAS ver. 9.0 (SAS Institute, Cary, NC). Significant linear trends were present where $p \leq 0.05$.

Authentic standards were purchased from Sigma–Aldrich (Milwaukee, WI) including 1-butanol, 1-hexanol, 1-pentanol, 1-propanol, 2-ethyl-1-hexanol, 2-furancarboxaldehyde, 2-methylbutyl acetate, 2-methyl-1-butanol, 2-methyl-1-propanol, 2-methylbutyl-2-methylbutanoate, 2-methylpropyl acetate, 2-propanol, 6-methyl-5-hepten-2-one, 1-methoxy-4-(2-propenyl)-benzene, butanal, butyl-2-methylbutanoate, butyl butanoate, butyl hexanoate, butyl propanoate, decanal, ethanol, ethyl-2-methylbutanoate, ethyl acetate, ethyl hexanoate, ethyl octanoate, ethyl propanoate, heptanal, hexanal, hexyl-2-methylbutanoate, hexyl acetate, hexyl butanoate, hexyl hexanoate, hexyl propanoate, methyl butanoate, methyl-2-methylbutanoate, nonanal, octanal, pentyl acetate, propyl acetate, propyl propanoate, benzaldehyde, butyl acetate, ethyl butanoate, and pentanal. Acetic acid was purchased from Fisher Scientific (Pittsburg, PA). Pentyl butanoate and propyl hexanoate were synthesized by combining 10 mL of butanoic acid or hexanoic acid with 15 mL of 1-pentanol or 1-propanol, respectively, in a round bottom flask as previously described (Mattheis et al., 2005). All reagents for ester synthesis were purchased from Sigma–Aldrich.

2.3. Sequence analysis and gene-specific primer design

Full-length cDNA and EST sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi/) and EMBL (http://www.ebi.ac.uk/embl/) including AY707098, AY517491, AY512893 and AX025508, and 3 unigene clusters and 6 singlets from Malus unigene assembly version 2 from GDR (http://www.bioinfo.wsu.edu/gdr/). The sequence alignment of selected apple AAT genes was analyzed using Clustal W (http://www.ebi.ac.uk/clustalw), and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences for gene-specific primers were selected based on the result of multi-sequence alignment (Table 1). Gene-specific amplifications for all four AAT genes were confirmed based on

Table 1
PCR primer sequences

Gene	Accession or unigene no.	Position	Primer sequences (5′ to 3′)
MdAAT1	AY707098	525–545 1376–1400	Forward TGATGCAGCTGGATTGCTCT Reverse CACTTACATCATTGACTAGTTGATC
MdAAT2	AY517491	719–741 1420–1440	Forward GTGATTGATCATTCTGATGGTTT Reverse CATCATTGACATGATCCTAGTTG
MdAAT3	Contigs 10730	711–732 1471–1496	Forward TGATTGATCATTCTGATGGCTC Reverse AACTCTACATA CAACTGATACAAC
MdAAT4	Contig 15415	302–320 706–727	Forward ACGTCGCTGCCATGTTCAC Reverse GCCAGGATCTGATGGTATTCTC
MdACS1	L31347	530–553 1529–1550	Forward ACTACCCAGGATTTGATAGAGAC Reverse GGTTTCTTGTACTCAGACCAGG
MdACS3	U73816	561–581 1226–1246	Forward TAGAGATTTGAGGTGGAGGAC Reverse CACTTCATGCAGCATGGAATC
MdACT	Contig 8474	740–759 1524–1543	Forward TTGCAGGTCGTGATCTGACT Reverse TACCAAGAGACTTGCAGAGG

sizes and sequence of each amplicon. Gene specific primers for MdACS1 and MdACS3 were designed from MdACS1 (L31347) and MdACS3 (U73816) based on sequence alignment analysis for the ACS gene family. Actin primers were designed from the Malus unigene contig 8474 of Malus unigene assembly version 2.

2.4. RNA isolation

Total RNA isolation was performed as described by Gasic et al. (2004) with modification. Peel tissue (2–3 g) was ground in liquid nitrogen using a baked mortar and pestle. Ground powder was transferred to 50-mL polypropylene tubes with 10 mL 2× CTAB extraction buffer. Tubes were briefly vortexed and then incubated at 60 °C for 15 min. After incubation, samples were homogenized using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) operated at 30,000 rpm. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added and the mixture vortexed for 2 min. The mixture was then centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant transferred to a clean tube. After repeating chloroform extraction process, the supernatant was transferred to 15 mL tubes where one third volume of 7.5 M LiCl was added, the tubes were inverted for mixing and then incubated at 4 °C for 16 h. Following incubation, the tubes were centrifuged at 14,000 × *g* for 30 min at 4 °C. The supernatant was discarded and the RNA pellet was washed with 750 μL 70% ethanol, the RNA was suspended in 150 μL DEPC water then stored at −80 °C. Aliquots of total RNA were treated with DNase I (Qiagen, Valencia, CA) and further purified using Qiagen RNeasy columns (Qiagen). Total RNA concentration was determined using a ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality verified by agarose gel electrophoresis.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

Synthesis of cDNAs from 2 μg of total RNA was performed in a 20-μL reaction using the Promega reverse transcription system (Promega, Madison, WI) with oligo dT as the primer. RT reaction mixture was diluted four times and 1 μL aliquot was used for PCR amplification. A set of apple actin primers was used in PCR reaction as the internal loading control. The PCR cycling conditions were: denature at 95 °C for 3 min, 30 cycles at 95 °C for 30 s each; 58 °C for 45 s, 72 °C for 60 s followed by a 7 min extension. PCR products (10 μL from 25 μL total PCR reactions) were resolved using a 1.5% agarose gel in 1× TAE buffer at 75 V for 1 h. The final gel image was recorded using a gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

3. Results

3.1. Sequence alignment and generation of gene-specific primers

The sequences of four full-length cDNAs, 3 unigene clusters and 6 singlets were analyzed to determine which represent fruit tissue expressed AAT genes and to design gene specific primers based on sequence polymorphism. The overall nucleotide sequences of the four full-length AAT cDNAs were highly homologous, particularly in the 5′ half (data not shown), and polymorphism around or at the 3′ untranslated region suggested that the AAT cDNAs can be represented by two genes, MdAAT1 (AY707898) and MdAAT2 (AY517491). None of the 6 singlets are expressed in fruit tissue; therefore, only 3 unigenes were analyzed further. Sequences for contig 18586 and MdAAT1 differ only by two single nucleotides and one di-nucleotide and therefore were considered to be the same gene. Contig 10730 has several indels in the 3′ untranslated regions compared with those from MdAAT1 and MdAAT2, and therefore was designated as MdAAT3 (Fig. 1). Contig 15415 was a short sequence and more divergent from the other three AAT genes and was designated as MdAAT4. Deletions and/or insertions at the 3′ ends of the first three apple AAT genes were employed to synthesize gene-specific primers, and a pair primers for an apple actin gene (MdACT, contig 8474) were used for ensuring equal loading of total RNA (Table 1).

3.2. Volatile emission during maturation and ripening

Starch index, firmness, ethylene production, and respiration rate indicated the onset of the climacteric ripening occurred in 'Golden Delicious' and 'Granny Smith' apples prior to 156 and 177 DAFB, respectively (Table 2). At harvest, volatile compounds detected were qualitatively and quantitatively different between cultivars (Tables 3 and 4). With the exception of propanal, (*E*)-2-hexenal, and 2-furancarboxaldehyde, production of all volatile compounds detected from 'Golden Delicious' apples increased over the course of the harvest period. Ethanol was the most prevalent compound detected at all harvest dates for 'Golden Delicious'. Increased production of most volatile compounds at 2 weeks after commercial maturity was coincident with an increase in internal ethylene concentration. The 'Granny Smith' volatile profile was composed primarily of hexanal and ethanol. Ester emission remained relatively low throughout the 'Granny Smith' harvest period, and emission of many esters did not change over the 8-week sampling period.

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AAT1/AY707098      TTTCAGCAGGAACTAGAGAGGATTACTCAGGAACCTAAGGAGGATATATG 1364
AAt2/AY517491      TTTCAGCAGGAACTAGAGAGGATTACTCAGGAACCTAAGGAGGATATATG 1407
AAT3/Contig10730   TTTCAGCAGGAACTAGAGAGGATTACTCAGGAACCTAAGGAGGATATATG 1399
Aat4/Contig15415   --------------------------------------------------

AAT1/AY707098      TAACAACCTTAGATCAACTAG-------TCAATGATGTAAGTGTTAAACG 1407  R1
AAT2/AY517491      TAACAACCTTAGATCAACTAGGATCATGTCAATGATGTAAGTGTTAAACG 1457  R2
AAT3/Contig10730   TAACAACCTTAGATTAACTAGGATCATGTCAATGATGTAAGTGTTAAACG 1449
AAT4/Contig15415   --------------------------------------------------

AAT1/AY707098      TAATGCACTTTCTGTA-------------------ATGTAGAGTTGTGT 1437
AAT2/AY517491      TAATGCACTTTCTGCA-------------------ATGTAGAGTTGTGT 1487
AAT3/Contig10730   TAATGCACTTTCTGTTGGTTAGTTGTATCAGTTGTTATGTAGAGTTGTGT 1499  R3
AAT4/Contig15415   --------------------------------------------------

AAT1/AY707098      CTCTTGGAACTTATCCAAGAGTTATAGCTGTTATCCAAAGGTCTGAATGT 1487
AAT2/AY517491      CTCTTGGAACTTATCCAAGAGTTATAGCTGTTATCCAAAGGTTTGAATGT 1537
AAT3/Contig10730   CTTTTGGAATTAATCCAAGACTTATGGGTGTTATGAAAAGGTTTCATTGT 1549
AAT4/Contig15415   --------------------------------------------------

AAT1/AY707098      TATTAAAAAATAGCCAATAATAAGATTGGCCTAGTGTTGTAATAAAAAAT 1537
AAT2/AY517491      TATTAAAAAATAGCCAATAATAAGATTGGCCTAGTGTTGTAATAAAAAAT 1587
AAT3/Contig10730   TATTAAAAA-TAGCCAA-AACAAGAT-GGCCTAGTGTTGTAATAAAAA-T 1595
AAT4/Contig15415   --------------------------------------------------

AAT1/AY707098      TGAAAGCAATAAAAT---TAAATTTCAGTTGCCGCTCAAAAAAAAAAAAA 1584
AAT2/AY517491      TGAAAGC---AAAAG---TAAACTAAA----------AAAAAAAAAAAAA 1621
AAT3/contig10730   TGAAAGCAACAAAGTATGTTAACTTCAGTTCC----------------- 1627
AAT4/Contig15415   --------------------------------------------------

AAT1/AY707098      AAAAAAA 1591
AAT2/AY517491      AAAAAAA 1628
AAT3/Contig10730   -------
AAT4/Contig15415   -------
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Fig. 1. Multiple sequence alignment for selected Malus AAT genes at 3′ end of nucleotide sequences. Sequence alignment was performed using Clustal W. The gene-specific reverse primer sequences for AAT1, AAT2 and AAT3 are underlined, and the stop codon for each gene is bolded. The position of the forward primers for four AAT genes and the reverse primers for AAT4 are not shown due to the size of the sequence alignment files. AAT4 sequence at 3′ end was not available.

1-MCP treatment reduced volatile production during fruit postharvest ripening at 20 °C (Tables 5 and 6). Emission of butyl-, 2-methylbutyl-, and hexyl acetates by 'Golden Delicious' was higher than other esters and this trend was similar in 1-MCP-treated fruit. Ethanol production was higher than other alcohols of both control and 1-MCP-treated fruits. Hexanal and ethanol production from 'Granny Smith' remained high from both control and 1-MCP-treated fruits. After 2 weeks at 20 °C, hexanal emission by 1-MCP-treated

Table 2
Maturity indicators for 'Golden Delicious' and 'Granny Smith' apples

Cultivar	Samples	DAFB	Starch index (1–6)	Firmness (N)	IEC ($\mu L\,L^{-1}$)	CO_2 production (μ mol $kg^{-1}\,s^{-1}$)
Golden Delicious	GD−4	128	1.4 ± 0.2	78.1 ± 4.4	0.03 ± 0.09	47 ± 6
	GD−2	142	1.8 ± 0.2	71.5 ± 4.0	0.01 ± 0.02	33 ± 3
	GD0	156	2.9 ± 1.0	71.6 ± 5.7	17 ± 33	42 ± 28
	GD+2	170	4.9 ± 0.7	68.1 ± 4.0	90 ± 88	31 ± 3
	GD+4	184	5.9 ± 0.9	56.1 ± 7.6	11 ± 34	97 ± 8
Granny Smith	GS−4	149	1.3 ± 0.2	89.4 ± 6.7	0.06 ± 0.15	28 ± 6
	GS−2	163	1.9 ± 0.6	84.1 ± 5.8	0.03 ± 0.03	50 ± 3
	GS0	177	2.4 ± 0.2	80.2 ± 5.3	0.12 ± 0.14	31 ± 6
	GS+2	191	3.0 ± 0.3	78.8 ± 4.7	2.4 ± 5.4	72 ± 8
	GS+4	205	5.6 ± 6.5	76.5 ± 5.3	33 ± 29	150 ± 15

Analyses were performed the day of harvest. Date of harvest expressed as days after full bloom (DAFB). Starch index values indicate full (1) or no (6) staining of cortex tissues by an I–KI solution. IEC: Internal ethylene concentration. Values are means plus standard deviation, $n = 18$ for starch, Newtons, and IEC, $n = 4$ for CO_2 production.

Table 3
Volatile aldehydes, alcohols, and esters detected from 'Golden Delicious' apples at harvest

Compound	Days after full bloom					
	128	142	156	170	184	
Propanal	0.05	0.22	0.03	1.80	nd	ns
Butanal	0.05	0.12	0.01	1.30	0.21	*
Pentanal	0.02	0.09	0.02	0.75	1.20	*
Hexanal	0.31	0.61	0.12	2.60	3.90	*
Heptanal	0.03	0.07	0.02	0.69	0.92	*
(*E*)-2-Hexenal	0.09	0.07	nd	nd	nd	ns
Octanal	0.04	0.10	0.03	0.92	1.60	*
Nonanal	0.07	0.15	0.05	1.40	2.40	*
Decanal	0.07	0.15	0.05	1.70	3.20	*
Benzaldehyde	0.08	0.11	0.04	0.34	1.20	*
2-Furancarboxaldehyde	0.01	0.03	nd	nd	0.15	ns
Isopropanol	0.26	0.49	0.05	6.20	2.10	*
Ethanol	8.67	12.7	3.75	118	237	*
1-Propanol	0.02	1.30	0.03	4.80	9.30	*
2-Methyl-1-propanol	0.02	0.01	0.01	0.58	0.83	*
1-Butanol	0.03	0.06	0.18	2.20	13.0	*
2-Methyl-1-butanol	0.01	0.01	0.04	0.49	1.40	*
1-Pentanol	0.01	0.02	0.01	0.17	0.31	*
1-Hexanol	0.06	0.05	0.03	0.39	1.00	*
Ethyl acetate	0.01	0.03	0.02	0.33	0.17	*
Propyl acetate	nd	nd	0.01	0.39	4.4	*
2-Methylpropyl acetate	0.01	0.01	0.02	0.30	2.4	*
Propyl propanoate	nd	nd	nd	0.01	0.14	*
Ethyl-2-methylbutyrate	nd	nd	nd	nd	0.01	*
Butyl acetate	0.03	0.06	0.35	6.2	92	*
2-Methylbutyl acetate	0.04	0.04	0.23	4.7	23	*
Butyl propanoate	nd	nd	0.01	0.39	2.9	*
Pentyl acetate	0.01	0.01	0.11	0.85	3.1	*
Butyl butyrate	0.01	0.01	0.04	1.1	3.9	*
Butyl-2-methylbutyrate	0.01	0.01	0.01	0.42	3.3	*
Ethyl hexanoate	nd	nd	nd	nd	0.02	*
Hexyl acetate	0.13	0.18	0.34	4.1	25	*
2-Methylbutyl-2-methylbutyrate	nd	0.01	nd	0.03	0.14	*
Propyl hexanoate	nd	nd	nd	0.01	0.05	*
Hexyl propanoate	0.01	0.01	0.01	0.19	0.85	*
Butyl hexanoate	0.02	0.01	0.01	0.32	1.2	*
Hexyl butyrate	0.02	0.02	0.04	0.47	1.3	*
Hexyl-2-methylbutyrate	0.03	0.10	0.02	0.79	2.0	*
Hexyl hexanoate	0.02	0.03	0.01	0.09	0.22	*
Aldehyde total	0.82	1.7	0.36	12	15	*
Alcohol total	9.1	14.6	4.1	130	270	*
Ester total	0.33	0.49	1.2	21	170	*
Total	10.3	16.8	5.7	163	455	*

Dynamic headspace samples were collected onto solid sorbent traps from fruit (~1 kg) held in sealed gas jars purged with compressed air. Trap contents were analyzed by GC/MS and identified by comparison of spectra with the NIST library, and by retention index and spectra comparisons with authentic standards. Values (nmol $kg^{-1}\,L^{-1}$) are means ($n = 3$). Significant (*, if $p \leq 0.05$) or non-significant (ns) linear regression for each compound evaluated by days after full bloom. nd: Not detectable, amount present below detection limit or not present.

Table 4
Volatile aldehydes, alcohols, and esters detected from 'Granny Smith' apples at harvest

Compound	Days after full bloom					
	149	163	177	191	205	
Propanal	0.04	0.08	0.14	0.10	nd	ns
Butanal	0.03	0.03	0.07	0.06	nd	ns
Pentanal	0.03	0.03	0.08	0.07	0.08	*
Hexanal	14	23	23	23	25	*
Heptanal	0.01	0.04	0.08	0.07	1.4	*
(*E*)-2-Hexenal	0.11	0.15	0.14	0.04	0.05	*
Octanal	0.02	0.03	0.14	0.11	0.04	*
Nonanal	0.03	0.04	0.17	0.18	0.07	*
Decanal	0.03	0.04	0.17	0.30	0.11	*
Benzaldehyde	0.02	0.02	0.08	0.10	0.09	*
2-Furancarboxaldehyde	0.01	nd	0.01	nd	0.01	ns
Isopropanol	0.07	0.12	0.59	1.8	0.48	*
Ethanol	3.9	5.1	8.6	11	13	*
1-Propanol	0.38	0.76	2.8	0.07	0.23	ns
2-Methyl-1-propanol	nd	nd	0.18	0.02	0.17	ns
1-Butanol	0.02	0.02	0.12	0.04	0.23	*
2-Methyl-1-butanol	0.01	0.02	0.03	0.28	1.2	*
1-Pentanol	nd	1.1	0.58	2.1	0.26	*
1-Hexanol	0.03	0.03	0.07	0.04	0.22	*
Ethyl acetate	0.01	0.01	0.01	0.02	0.04	*
Ethyl propanoate	nd	nd	nd	nd	3.2	*
Propyl acetate	0.01	0.02	0.03	0.04	0.07	*
Methyl butyrate	nd	nd	nd	nd	0.02	*
2-Methyl-2-methylbutyrate	nd	nd	nd	0.01	0.03	*
2-Methylpropyl acetate	nd	nd	0.01	0.01	0.01	*
Ethyl butyrate	0.01	0.01	0.01	0.06	0.07	*
Propyl propanoate	nd	nd	0.01	0.01	0.09	*
Ethyl-2-methylbutyrate	nd	nd	0.01	0.01	0.03	*
Butyl acetate	0.02	0.04	0.07	0.02	0.57	*
2-Methylbutyl acetate	nd	nd	0.001	nd	nd	ns
Propyl butyrate	nd	nd	nd	0.01	0.10	*
Butyl propanoate	0.01	0.01	nd	0.01	0.07	ns
Pentyl acetate	0.02	0.01	0.02	0.02	0.04	ns
Butyl butyrate	0.03	0.01	0.01	0.01	0.02	ns
Butyl-2-methylbutyrate	0.02	0.01	0.01	0.01	0.01	ns
Ethyl hexanoate	nd	nd	0.01	nd	0.01	ns
Hexyl acetate	0.28	0.17	0.38	0.10	1.1	ns
2-Methylbutyl-2-methylbutyrate	0.01	0.02	0.01	0.01	0.01	ns
Propyl hexanoate	nd	0.01	0.03	0.01	0.01	*
Hexyl propanoate	0.01	0.01	0.02	0.02	0.07	*
Butyl hexanoate	0.02	0.02	0.05	0.01	0.03	ns
Hexyl butyrate	0.02	0.01	0.05	0.03	0.09	*
Hexyl-2-methylbutyrate	0.06	0.04	0.08	0.07	0.05	ns
Ethyl octanoate	0.01	0.01	0.01	0.01	0.03	ns
Aldehyde total	15	24	24	24	26	*
Alcohol total	4.5	7.1	13	15	16	*
Ester total	0.6	0.4	0.8	0.4	5.9	*
Total	20.1	31.5	37.8	39.4	47.9	*

Dynamic headspace samples were collected onto solid sorbent traps from fruit (~1 kg) held in sealed glass jars purged with compressed air. Trap contents were analyzed by GC/MS. Values are means ($n = 3$). Significant (*, if $p \leq 0.05$) or non-significant (ns) linear regression for each compound evaluated by days after full bloom. nd: Not detectable, amount present below detection limit or not present.

Table 5
Volatile aldehydes, alcohols, and esters detected from 'Golden Delicious' apples held at 20 °C after harvest

Compound	Weeks at 20 °C after harvest					
	2		4		6	
	Control	1-MCP	Control	1-MCP	Control	1-MCP
Propanal	3.3*	1.1	nd	nd	nd	nd
Butanal	2.4*	0.85	0.18*	nd	nd	nd
Pentanal	1.3*	0.42	1.8*	0.57	0.14*	0.05
Hexanal	4.5*	1.4	8.7*	2.4	0.64	0.22
Heptanal	2.2*	0.45	2.3*	0.40	0.35*	0.05
Octanal	2.8*	0.41	2.6*	1.1	0.14	0.04
Nonanal	4.9*	0.69	4.5*	1.6	0.20	0.18
Decanal	6.6*	0.59	7.5*	2.5	0.27*	0.07
Benzaldehyde	1.4*	0.16	2.7*	0.86	0.09	0.05
2-Furancarboxaldehyde	0.19	nd	0.74*	0.03	0.02	0.01
Isopropanol	8.3	4.0	3.2*	1.9	0.72*	0.20
Ethanol	210*	70	510*	200	52*	20
1-Propanol	9.2*	3.3	30*	3.2	11*	0.15
2-Methyl-1-propanol	0.89*	0.30	0.77*	0.10	0.22*	nd
1-Butanol	19*	1.4	28*	0.36	11*	2.0
2-Methyl-1-butanol	30*	0.25	4.5*	0.09	1.8*	0.17
1-Pentanol	0.49*	0.05	0.53*	0.12	0.12*	0.03
1-Hexanol	2.1*	0.17	2.0*	0.16	0.73*	0.27
Ethyl acetate	0.93*	0.24	0.97*	0.09	0.24*	nd
Ethyl propanoate	nd	nd	nd	nd	0.08*	nd
Propyl acetate	5.2*	0.45	52	nd	29*	0.25
2-Methylpropyl acetate	0.90*	0.07	1.0*	0.01	0.71*	0.07
Ethyl butyrate	nd	nd	nd	nd	0.01	nd
Propyl propanoate	0.31*	0.03	3.4	nd	2.1*	0.01
Ethyl-2-methylbutyrate	0.01*	nd	0.02*	nd	0.01	nd
Butyl acetate	81*	6.0	131*	0.17	72	13
2-Methylbutyl acetate	44*	3.6	49*	0.29	18	2.1
Butyl propanoate	2.2*	0.50	9.5*	nd	3.0*	0.26
Pentyl acetate	4.3*	0.33	3.9*	0.02	1.3	0.62
Butyl butyrate	3.8*	0.28	2.8*	0.01	0.51	0.28
Butyl-2-methylbutyrate	3.3*	0.28	3.0*	0.01	0.28*	0.07
Ethyl hexanoate	nd*	0.01	nd	nd	0.01	nd
Hexyl acetate	43*	3.5	37*	0.36	9.0*	4.4
2-Methylbutyl-2-methylbutyrate	0.04*	0.01	0.15*	nd	0.02	0.01
Propyl hexanoate	0.14*	0.01	0.77*	nd	0.08*	0.01
Hexyl propanoate	2.9*	0.23	3.4*	nd	0.64*	0.15
Butyl hexanoate	1.6*	0.13	2.7*	0.02	0.16	0.08
Hexyl butyrate	1.7*	0.13	2.6*	0.02	0.23	0.18
Hexyl-2-methylbutyrate	3.1*	0.25	5.5*	0.04	0.35	0.16
Hexyl hexanoate	0.30*	0.02	1.6*	0.02	0.05	0.02
Aldehyde total	29.6*	6.1	31*	9.4	1.9	0.68
Alcohol total	260*	79	580*	210	78*	23
Ester total	200*	16	310*	1.0	140*	21
Total	490*	101	920*	220	218*	44

Dynamic headspace samples were collected onto solid sorbent traps from fruit (~1 kg) held in sealed gas jars purged with compressed air. Trap contents were analyzed by GC/MS. Values are means ($n = 3$). Treatment means compared using paired t-test within ripening periods, "*" indicates significant differences, with $p \leq 0.05$. nd: Not detectable, amount present below detection limit or not present.

fruit was higher than control fruit. Control fruit ester production following 4 and 6 weeks post-storage ripening was significantly higher than 1-MCP-treated fruit. Ethyl propanoate, ethyl butyrate, and ethyl-2-methylbutyrate were the most prevalent esters produced by 'Granny Smith' during ripening.

3.3. Expression patterns of apple AAT and ACS genes during on-tree fruit ripening

Cultivar-specific AAT gene expression patterns were evident for 'Golden Delicious' and 'Granny Smith' apples during maturation and on-tree ripening (Fig. 2). Expression levels were higher overall in 'Golden Delicious' compared to 'Granny Smith' for all four AAT genes at all harvests, which was consistent with observed total volatile ester emission (Tables 3 and 4). MdAAT1 and MdAAT2 appeared to be the predominantly expressed genes in both cultivars, and their expression level increased as ripening progressed. The transcript levels of AAT3 and AAT4 decreased at or after the onset of ripening. MdAAT1 and MdAAT2 expression was highest as climacteric ripening began with the detection of significantly increased MdACS1 transcripts. Detection of the MdACS1 transcript was coincident with increased IEC in 'Granny Smith' at H+4, but MdACS1 was detectable at a low level before H+4 in 'Golden Delicious'. MdACS3 transcript was present from samples at all harvest dates and the expression level increased as ripening progressed in 'Golden Delicious', however it is barely detectable in 'Granny Smith' until the later harvests.

3.4. Expression patterns of apple AAT and ACS genes during postharvest ripening

Expression of MdAAT1 and MdAAT2 was stronger in 'Golden Delicious' than in 'Granny Smith' during postharvest ripening at 20 °C (Fig. 3). MdAAT1 expression remained constant in 'Golden

Table 6
Volatile aldehydes, alcohols, and esters detected from 'Granny Smith' apples held at 20 °C after harvest

Compound	Weeks at 20 °C after harvest					
	2		4		6	
	Control	1-MCP	Control	1-MCP	Control	1-MCP
Propanal	nd	nd	nd	nd	nd	nd
Butanal	nd	nd	nd	nd	nd	nd
Pentanal	nd*	0.19	0.86*	0.31	0.56*	0.12
Hexanal	16*	43	180*	56	170*	43
Heptanal	0.09	0.09	9.0*	0.08	2.2*	0.05
(*E*)-2-Hexenal	0.04	0.01	nd*	0.10	nd*	0.01
Octanal	0.11	0.11	0.46*	0.03	0.16*	0.11
Nonanal	0.12*	0.19	0.61*	0.18	0.53*	0.15
Decanal	0.20*	0.04	0.75*	0.23	0.73*	0.19
Benzaldehyde	0.10*	0.13	1.1*	0.31	0.19*	0.03
2-Furancarboxaldehyde	0.01*	0.01	0.05*	0.02	nd*	nd
Total aldehydes	17*	44	193*	58	175*	43
Isopropanol	0.16*	1.4	7.2*	1.7	1.3*	0.15
Ethanol	2.7*	9.4	121*	18	196*	7.1
1-Propanol	0.09*	0.05	3.3*	0.53	4.2*	nd
2-Methyl-1-propanol	0.08*	0.29	1.0*	0.12	0.64*	0.05
1-Butanol	0.16*	0.07	2.5*	0.09	2.1*	0.02
2-Methyl-1-butanol	0.60*	0.20	14*	0.04	6.3*	0.02
1-Pentanol	1.6*	0.67	30*	31	4.3*	0.48
1-Hexanol	0.20*	0.03	5.9*	0.10	2.7*	0.05
Total alcohols	5.6*	12	185*	51	218*	7.9
Total	1.8*	1.1	160*	0.97	87*	0.33
Ethyl acetate	nd	nd	0.10*	0.02	0.12	0.02
Ethyl propanoate	0.01	0.01	39*	0.01	35*	0.01
Propyl acetate	nd*	0.02	0.04	nd	0.13*	nd
Methyl butyrate	nd	nd	1.3*	nd	0.56*	nd
Methyl-2-methylbutyrate	nd	nd	0.98*	nd	0.16*	0.01
2-Methylpropyl acetate	nd	nd	nd	nd	nd	nd
Ethyl butyrate	0.09*	0.01	63*	0.04	35*	0.02
Propyl propanoate	0.02	0.01	4.0*	0.01	5.2*	nd
Ethyl-2-methylbutyrate	0.04	0.01	28*	0.01	6.5*	0.01
Butyl acetate	0.07	0.12	0.22	0.12	0.23*	0.05
2-Methylbutyl acetate	0.01	0.02	0.01	0.01	0.01	nd
Propyl butyrate	0.07	0.01	4.2*	0.01	1.0*	0.01
Butyl propanoate	0.08	0.02	0.53*	nd	0.39*	0.01
Pentyl acetate	0.02	0.01	0.15*	0.02	0.09	0.02
Butyl butyrate	0.03	0.03	0.12*	0.01	0.04	0.01
Butyl-2-methylbutyrate	0.02	0.02	0.13*	0.01	0.01	0.01
Ethyl hexanoate	0.02	nd	2.9*	nd	1.01*	0.01
Hexyl acetate	0.74	0.36	2.0	0.38	0.32	0.09
2-Methylbutyl-2-methylbutyrate	0.02	0.01	0.12	0.01	0.01	nd
Propyl hexanoate	0.02	0.01	0.38*	0.01	0.02	nd
Hexyl propanoate	0.11	0.03	0.41*	0.02	0.04	0.01
Butyl hexanoate	0.10	0.16	0.24	0.08	0.01	0.01
Hexyl butyrate	0.13	0.11	0.54*	0.06	0.02	0.02
Hexyl-2-methylbutyrate	0.18	0.15	1.2*	0.08	0.04	0.02
Ethyl octanoate	0.02	0.01	0.11	0.02	0.07	0.01
Hexyl hexanoate	0.01	0.01	0.01	0.01	0.01	nd
Aldehyde total	17*	44	190*	58	175*	43
Alcohol total	5.6*	12	190*	51	218*	7.9
Ester total	1.8	1.1	160*	1	87*	0.3
Total	24*	57	540*	110	480*	51

Dynamic headspace samples were collected onto solid sorbent traps from fruit (~1 kg) held in sealed gas jars purged with compressed air. Trap contents were analyzed by GC/MS. Values are means ($n = 3$). Treatment means were compared using paired *t*-test within ripening periods, "*" indicates significant differences, with $p \leq 0.05$. nd: Not detectable, amount present below detection limit or not present.

Delicious' but decreased in 'Granny Smith'. MdAAT3 and MdAAT4 transcripts were undetectable during the postharvest ripening period. Inhibition of ethylene action by 1-MCP treatment did not appear to result in altered MdAAT1 expression in either cultivar. However, expression of MdAAT2 was suppressed in 'Golden Delicious' and increased slightly in 'Granny Smith' following 1-MCP treatment. MdACS1 transcripts in both cultivars were not detected in 1-MCP-treated fruit after 2 and 4 weeks, but expression recovered at 6 weeks in both cultivars. The expression of MdACS3 in 'Golden Delicious' was not affected by 1-MCP treatment, while in 'Granny Smith', MdACS3 was detectable only in control fruit at 4 or 6 weeks after harvest.

4. Discussion

Production of aromatic volatile ester varies significantly among apple cultivars (López et al., 1998a; Holland et al., 2005), while the genetic controls underlying these differences have not been elucidated. AAT catalyzes the last step of volatile ester biosynthesis in apple (Souleyre et al., 2005; Li et al., 2006) and other fruits (Aharoni et al., 2000; El-Sharkawy et al., 2005), and ethylene is required for the AAT gene expression (Defilippi et al., 2005a; Schaffer et al., 2007). In this study, gene expression patterns were characterized for AAT and ACS gene family members between 'Golden Delicious' and 'Granny Smith', two phenotypic extremes

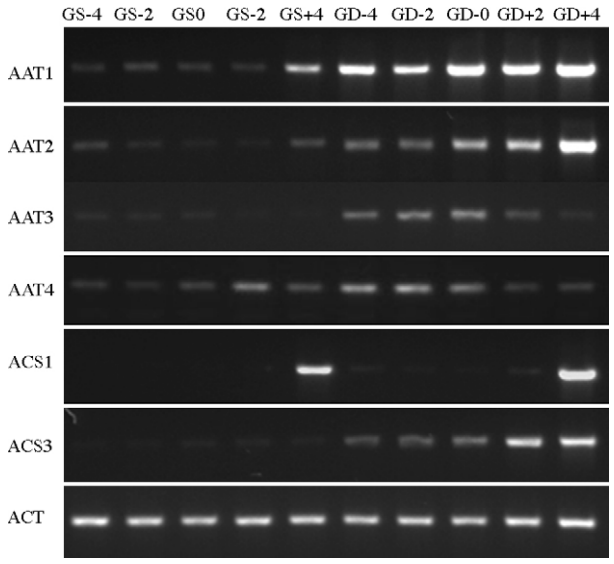

Fig. 2. Expression patterns of four AAT genes and two ACS genes in 'Granny Smith' and 'Golden Delicious' apple peel tissue at different ripening stages. Fruit peel tissues with 2-week intervals beginning 128 (GD−4) or 149 (GS−4) days after full bloom (DAFB) for 'Golden Delicious' (GD), 'Granny Smith' (GS), respectively. Top axis label indicates cultivar and weeks prior to (designate "−"), at (0), or after (+) physiological maturity was attained.

with respect to volatile ester emission. Higher expression of all four AAT genes in the high ester producing cultivar 'Golden Delicious' compared to the low ester producing cultivar 'Granny Smith' suggests the expression of AAT genes contributes to the phenotypic difference between these two cultivars. Between these two cultivars, the differences on AAT gene expression intensity, particularly for AAT1 and AAT2, are in principal correlated with the levels of total detected emission of volatile esters (Tables 3–6).

A requirement for ethylene and ethylene action to stimulate ripening-related volatile biosynthesis in apple fruit has been demonstrated through transgenic suppression of ethylene biosynthesis and chemical inhibition of ethylene action (Fan et al., 1998; Defilippi et al., 2005a; Schaffer et al., 2007). MdACS1 gene expression is known to be the rate limiting step in climacteric ethylene production in apple (Lau et al., 1986; Harada et al., 2000). The results from this study demonstrate that enhanced MdACS1 expression roughly coincides with autocatalytic ethylene production in these two apple cultivars. However, the MdACS3 transcript was detectable prior to onset of the climacteric in both cultivars although with different levels. As AAT gene transcripts were detected long before the expression of MdACS1, the results suggest that low-level ethylene production via the MdACS3 product (Wiersma et al., 2007), likely responsible for system I ethylene biosynthesis (Barry et al., 2000), could be responsible for the expression of AAT genes at an early stage of fruit development, and other ripening processes before the climacteric ripening starts due to the expression of ACS1. The differential effect of 1-MCP on expression of MdAAT1 and MdAAT2 reflect diverse regulation of these two genes by ethylene, or different requirement on ethylene concentration threshold. The unchanged MdACS3 expression following 1-MCP treatment in 'Golden Delicious' may allow a low level of ethylene production that is sufficient to support the continued expression of MdAAT1.

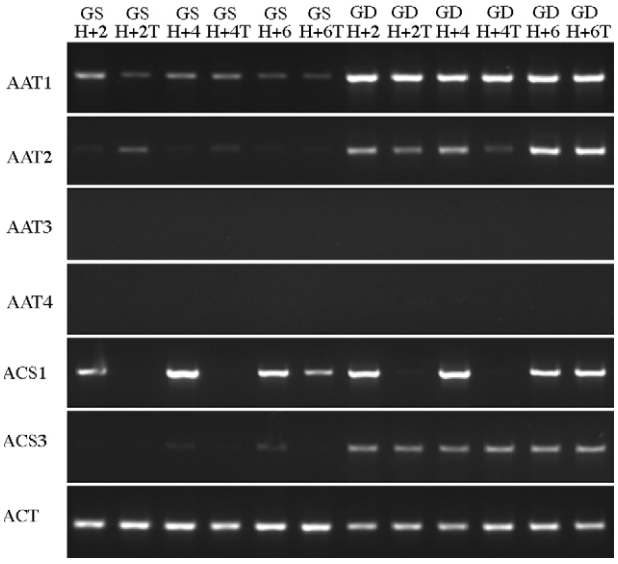

Fig. 3. Expression patterns of four AAT genes and two ACS genes in 'Granny Smith' and 'Golden Delicious' apple peel tissue during ripening after harvest. Fruit were harvested 156 (GD0) or 177 (GS0) days after full bloom (DAFB) and held up to 6 weeks at 20 °C. Top axis label indicates cultivar, weeks after harvest (H+2, H+4 and H+6), and "T" indicates treatment at harvest with 1-MCP.

Substrate availability, enzyme kinetics, and AAT isozyme substrate specificity could all contribute to volatile production phenotypes (Echeverría et al., 2004; Fellman et al., 1993). AAT isozymes have broad specificity for a variety of alcohol and acyl-CoA substrates (Aharoni et al., 2000; Souleyre et al., 2005). Both biochemical and transgenic studies suggest that fatty acid and amino acid metabolism generate ester precursors. Processes such as β-oxidation and hydroperoxy acid cleavage control the availability of primary precursors for fatty acid-derived esters (Song and Bangerth, 2003; Wang et al., 2001). Likewise, enzymes in upstream pathways, including pyruvate decarboxylase (PDC), alcohol dehydrogenase (ADH), and lipoxygenase (LOX), which catalyze reactions producing substrate from amino acids and fatty acids, could also contribute to the different ester composition exhibited by different apple cultivars (Bartley and Hindley, 1980; Ke et al., 1994; Rowan et al., 1999; Echeverría et al., 2004). In the current study, headspace alcohol concentrations were significantly higher in 'Golden Delicious' than in 'Granny Smith' demonstrating differences in precursor availability. As there is evidence that the substrates for AAT gene products are also under the influence of ethylene action (Schaffer et al., 2007), the role of MdACS3 gene expression at early fruit maturation stage cannot be dismissed since MdACS1 was expressed 4 weeks after commercial maturity. Alternatively, low-level ethylene production from MdACS3 expression, combined with other physiological stimuli, may increase substrate availability to a level that allosterically regulates AAT expression. Therefore, specifics of a relationship between cultivar-specific MdACS3 expression and volatile ester emission as well as other ripening processes remain to be elucidated.

Detachment of the fruit from tree appears to have different effect on the expression of AAT and ACS genes. For example, MdACS1 transcript levels were higher in the tissues which harvested at commercial maturity and stored for 2 weeks (both GS H+2 and GD H+2 in Fig. 3), compared with those of on-tree ripening fruits (GD+2 and GS+2 in Fig. 2). However, AAT gene expression, particularly MdAAT3 and MdAAT4, was suppressed at similar stages after fruit detachment. This demonstrates one way that harvest timing could lead to changes in the edible characteristics by altering the ripening and aroma synthesis patterns. Differential expression of all four AAT genes between 'Golden Delicious' and 'Granny Smith' may indicate the presence of specific cis- or trans-acting factors which render 'Golden Delicious' more sensitive to the basal level of ethylene by ACS3 expression prior to climacteric ripening. The fact that MdAAT1

and MdAAT2 are expressed as early as 4 weeks after full bloom in apple fruit (data not shown) as well as in floral and leaf tissues (Li et al., 2006) may suggest that AAT also catalyzes the biosynthesis of non-ripening-related ester compounds, which function in other biological processes.

The results from this study reveal associations between AAT and ACS gene expression and phenotypic variations in volatile ester production, and suggest that expression of AAT genes contributes to the variation in volatile ester emission detected between these two cultivars. The expression patterns of MdACS1 and MdACS3 over the maturation and ripening period indicate a potential role for MdACS3 to induce AAT gene expression early in the fruit maturation process. The disparity in MdACS1 and MdACS3 expression following fruit exposure to 1-MCP and the constitutive nature of MdACS3 expression in 'Golden Delicious' may suggest the role of MdACS3 on apple postharvest storability, and of 1-MCP treatment efficacy to different cultivars. To our knowledge, this is the first report of cultivar differences in AAT and ACS gene expression during maturation and ripening of apple fruit. For genomics-assisted breeding, elucidation of the genetic control mechanisms and identification of genes that regulate volatile ester biosynthesis may lead to the generation of functional molecular markers (Andersen and Lübberstedt, 2003; Varshney et al., 2005). The potential role of the expression of MdACS3 on the efficacy of 1-MCP treatment supports the idea that study of tree fruit genetics and genomics will not only facilitate genomics-assisted breeding but also benefit the development of postharvest management strategies.

Acknowledgments

The authors gratefully thank Ann Callahan and Tianbao Yang for their helpful comments. We thank Mallela Magana, Janie Countryman and David Buchanan for excellent technical assistance.

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